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Drosophila molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2☆

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Abstract Bursicon is a neurohumoral agent responsible for tanning and hardening of the cuticle and expansion of the wings during the final phase of insect metamorphosis. Although the hormonal activity was described more than 40 years ago, the molecular nature of bursicon has remained elusive. We identify here *Drosophila* bioactive bursicon as a heterodimer made of two cystine knot polypeptides. This conclusion was reached in part from the unexpected observation that in the genome of the honey bee, the orthologs of the two *Drosophila* proteins are predicted to be fused in a single open reading frame. The heterodimeric *Drosophila* protein displays bursicon bioactivity in freshly eclosed neck-ligated flies and is the natural agonist of the orphan G protein-coupled receptor DLGR2.

Keywords: Bursicon; G protein-coupled receptor; Leucine-rich repeats containing GPCR; Cystine knot; Comparative genomics; Glycoprotein hormone receptor

1. Introduction

Bursicon was described more than 40 years ago as a bioactivity present in CNS (central nervous system) extracts and hemolymph of insects after eclosion, capable of rescuing flies from defective tanning and sclerotization of the cuticle caused by neck-ligation [1–4]. It is a key regulator of the final steps in the stereotyped developmental and behavioural program of ecdysis, i.e., the shedding of the remains of the old cuticle and the consequent maturation of the new one. Bursicon is believed to act downstream of a sequence of regulatory peptides, such as ecdysis triggering hormone (ETH), eclosion hormone (EH) and crustacean cardioactive peptide (CCAP) [5–11]. Bursicon bioactivity can be partially mimicked by cyclic AMP analogs [12,13] and was recently shown to be involved in apoptotic removal of wing epidermal cells during the last step of metamorphosis into adult *Drosophila* [14]. Purification of bioactive bursicon from nerve cords of the cockroach *Periplaneta americana* yielded a 30 kDa protein [15] from which several tryptic peptides were obtained and micro-sequenced [16]. Comparison of these sequences with available genomic data identified a 522 nucleotide open reading frame (ORF) in *Drosophila* (CG13419) that codes for a cystine knot protein with close orthologs in the bee *Apis mellifera* and in the mosquito *Anopheles gambiae* [17,18] (Fig. 1A). The link between the *Drosophila* ORF and bursicon was established from the defective post-ecdysial behaviour of flies with loss-of-function mutations in the putative gene, and the corresponding decrease in bursicon bioactivity in mutant extracts [17]. Accordingly, it was proposed that bursicon would be a homodimeric 30 kDa protein made of two 15 kDa CG13419 mature protomers [17].

DLGR2 is a G protein-coupled receptor of *Drosophila* distantly related to the vertebrate glycoprotein hormone and relaxin receptors [19,20]. It has been proposed as the receptor for bursicon because flies with loss-of-function mutations in DLGR2 display a phenotype (*rickets* $rk^{-/-}$) which is similar to bursicon deficiency induced by neck-ligation. It consists in defective sclerotization and melanization of the cuticle, as well as abnormal wing expansion after adult eclosion. These mutants produce bioactive bursicon, and their phenotype cannot be rescued by bursicon injection [13].

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Abbreviations: ORF, open reading frame; LGR, leucine-rich repeats containing G protein-coupled receptor; CTCK, C-terminal cystine knot; WGS, whole-genome sequencing; Bursα, bursicon α subunit; Bursβ, bursicon β subunit

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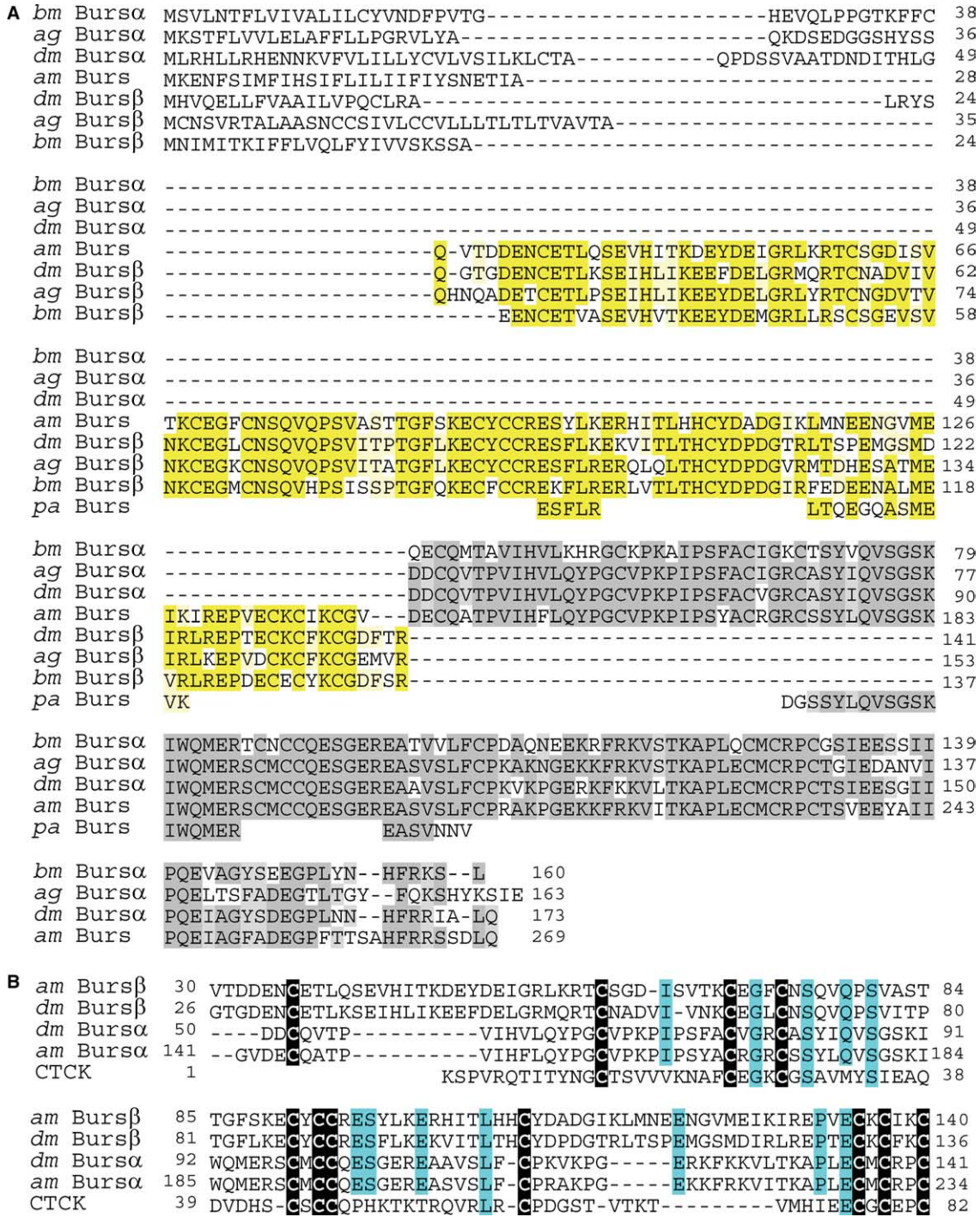

Fig. 1. Alignments of insect bursicon proteins. (A) CG13419 and CG15284 orthologs (called Bursα and Bursβ, respectively) from mosquito (*ag: Anopheles gambiae*), silkworm (*bm: Bombyx mori*) and fruit fly (*dm: Drosophila melanogaster*) were aligned along the bee (*am: Apis mellifera*) single-chain multidomain bursicon. The tryptic peptides identified in bursicon preparations from the cockroach (*pa: Periplaneta americana*), a heterometabolic insect, are also aligned. Conserved Bursα and Bursβ sequences are shaded in grey and yellow, respectively. Residues identical among 3, 4, or 5 species are dark shaded, while residues identical between two species are pale shaded. Silkworm Bursα (BN000691) and Bursβ (BN000690), mosquito Bursβ (BN000689) and bee single-chain bursicon (BN000692) were manually predicted on available WGS DNA sequences (see Section 2 for details). *Drosophila* CG13419 and CG15284 cDNAs were cloned, sequenced and annotated in EMBL/GenBank/DDBJ databases (Accession Nos. AJ862523 and AJ862524, respectively). The putative signal peptides are shown at the left side on the top of the figure. (B) Alignment of *Drosophila* Bursα and Bursβ proteins, together with the Bursα and Bursβ domains from bee single-chain bursicon, and a consensus cystine knot-like domain (CTCK: C-terminal cystine knot) [30]. Conserved cysteine residues are highlighted. Other conserved residues are shaded in blue.

In the present study we have used a combination of comparative genomic, biological and biochemical approaches to identify bioactive bursicon as a cystine knot heterodimeric protein and the natural agonist of DLGR2.

2. Methods

2.1. Similarity searches and protein alignments

Similarity searches of insect genomes and proteins were made on the NCBI platform (www.ncbi.nih.gov) by TBLASTN or BLASTP, respectively [21]. Multiple protein alignments were performed with CLUSTAL (www.clustalw.genome.jp) using default settings.

2.2. Protein predictions on WGS (whole-genome sequencing) DNA sequences

The bee, mosquito and silkworm genomes were analysed for the presence of *Drosophila* CG13419 or CG15284 orthologs with TBLASTN (*Anopheles* CG13419-like sequence was already present in the database; Accession No. AY735443). Because the N-termini are not conserved among species, the putative signal peptides could not be identified in the TBLASTN alignments. In consequence, each locus was individually screened for the presence of potential signal peptides [22] located in frame in the same exon with CG13419 or CG15284 homologous sequences (ORF finder tool at the NCBI, www.ncbi.nih.gov/gorf; SignalP 3.0 Server; www.cbs.dtu.dk/services/SignalP/). In the case of the silkworm CG13419 ortholog, the putative signal peptide was identified in a predicted separate exon, located 56 base pairs upstream from the starting point of CG13419-like segment. Exon prediction was performed at www.fruitfly.org/seq_tools/splice.html. Bee, silkworm and mosquito predicted sequences were submitted to EMBL/GenBank/DDBJ databases. For accession numbers see legend of Fig. 1.

2.3. PCR, cloning and sequencing

Drosophila cDNAs, normalized to the housekeeping *Rp49* (Ribosomal protein 49) cDNA, derived from different developmental stages and tissues ('Rapid-Scan™ Gene expression panel', Origene Technologies, Rockville, MD) were used as template to amplify bursicon α subunit (Bursα) and bursiconβ subunit (Bursβ) cDNAs by means of PCR with gene-specific primers [Bursα (cg13419): fwd gcggatcctcgcagtcgggccgacgagatg; rev gcgaattcagtgctaaactgatgggggaatcta. They contain a 5′ *Bam*HI and *Eco*RI restriction site (underlined), respectively. Annealing temperature 55 °C. Bursβ (cg15284): fwd atgcatgtccaggaactgctc; rev ttaacgtgtgaaatcgccaca. Annealing temperature 57 °C]. Both reactions were performed for 35 cycles. The resulting products were analysed by agarose gel electrophoresis, purified, cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) and sequenced (ABI Prism 310, Applied Biosystems, Foster City, CA). ORFs coding for the bursicon subunits were further subcloned into pcDNA3.1 (Invitrogen) for transfection of COS-7 cells.

2.4. Preparation of conditioned media

COS-7 cells growing in 35 mm culture dishes were transfected with 1 μg of plasmid DNA using the DEAE-dextran method [23]. The day after transfection, the culture medium (DMEM containing 10% Fetal Bovine Serum (FBS)) was replaced by the same medium with 1% FBS. Twenty-four hours later, the medium was recovered, centrifuged at $2000 \times g$ to eliminate cellular debris, immediately frozen in liquid nitrogen and stored at −20 °C until use. For dose–response curves and gel filtration chromatography, conditioned medium was concentrated using ultrafiltration devices with a 10 kDa MW cut-off (Amicon Ultra Centrifugal Filters, Millipore Corporation).

2.5. Stimulation of cAMP accumulation

Drosophila DLGR1, DLGR2 [19,28] and human hLGR4 and hLGR5 cloned in pcDNA3 were transfected in COS-7 cells in the same way as described above. Forty-eight hours after transfection, expression of the various receptors was assayed by flow immunocytometry, using a panel of monoclonal antibodies (not shown). Cells were washed once with Krebs–Ringer–HEPES (KRH) buffer and incubated at 37 °C for 30 min. Buffer was then replaced by 1 ml of fresh KRH supplemented with 10% of conditioned medium and 25 μM rolipram (a cAMP phosphodiesterase inhibitor, Laboratoire Logeais, Paris, France). After a 30 min incubation, the level of intracellular cAMP was measured by RIA as described previously [23].

2.6. Gel filtration chromatography

One millilitre of 20× concentrated culture medium from cells co-expressing recombinant CG13419 and CG15284 proteins was applied onto a gel filtration column (HILoad™ 16/60 Superdex™ 75 prep Grade, Amersham Biosciences, Freiburg, Germany). Calibrations and runs were made in $Na_3(PO_4)$ 20 mM/NaCl 200 mM buffer. Eluates corresponding to sizes between 70 and 10 kDa were collected in 2 ml fractions and frozen immediately in liquid nitrogen. One hundred, or 5 μl of each fraction were assayed for cAMP stimulating activity on COS-7 cells expressing DLGR2, or in the bursicon bioassay, respectively.

2.7. Bursicon bioassay

Bursicon bioactivity was determined by means of the flesh fly cuticle tanning bioassay [2]. Flies (*Neobellieria bullata*) were decapitated (or neck-ligated) after eclosion and kept at room temperature for 1.5 h in a humidified petri dish. This initial incubation period allowed for a pre-selection of flies (the vast majority) that did not display any endogenous bursicon activity after decapitation (or neck-ligation). Flies that remained pale were injected with 5 μl of solution (saline, CNS extract, 1 mM 8Br-cAMP, conditioned culture media or chromatographic fractions) and cuticular tanning was evaluated 3 h post-injection. Qualitative evaluation of tanning was as follows: flies do not tan: "Defective tanning"; only the abdomen tans: "Intermediate tanning"; both the abdomen and the thorax tan: "Full Tanning". The CNS extract was prepared from freshly eclosed flesh flies. CNS were dissected and homogenized in saline solution (127.7 mM NaCl, 4.7 mM KCl) and 0.25 CNS-equivalents were injected into each fly.

3. Results and discussion

3.1. Drosophila CG13419 has no Bursicon bioactivity and does not activate DLGR2

With the aim of establishing a direct experimental link between the cystine knot protein described by Dewey et al. [17] (CG13419, hereafter Bursα) and bursicon, we transfected COS-7 cells with Bursα cDNA inserted in an expression plasmid. The conditioned medium was tested for bursicon bioactivity in freshly eclosed, neck-ligated *N. bullata* (see Fig. 2C and Section 2), and for its ability to stimulate cAMP production in COS-7 cells expressing DLGR2 (Fig. 2A). Contrary to what was suggested on the basis of genetic data by Dewey et al. [17], Bursα was devoid of bursicon bioactivity and incapable of activating DLGR2. Together with the evidence from loss-of-function mutations of Bursα [17], these results indicated that a piece of information was lacking in the bursicon puzzle, most probably a dimerisation partner for Bursα.

3.2. Identification of a new Bursicon candidate

Honegger et al. [16] reported a set of peptide sequences from purified bursicon preparations of the cockroach *P. americana*. It is from some of these peptides that CG13419 (Bursα) was identified in the *Drosophila* genome [17].

Using the sequence of one of these peptides which was initially considered as a contaminant in the bursicon preparation (peptide sequence: ESFLR, see Fig. 1), we found a new ORF in *Drosophila* encoding a 15 kDa cystine knot protein

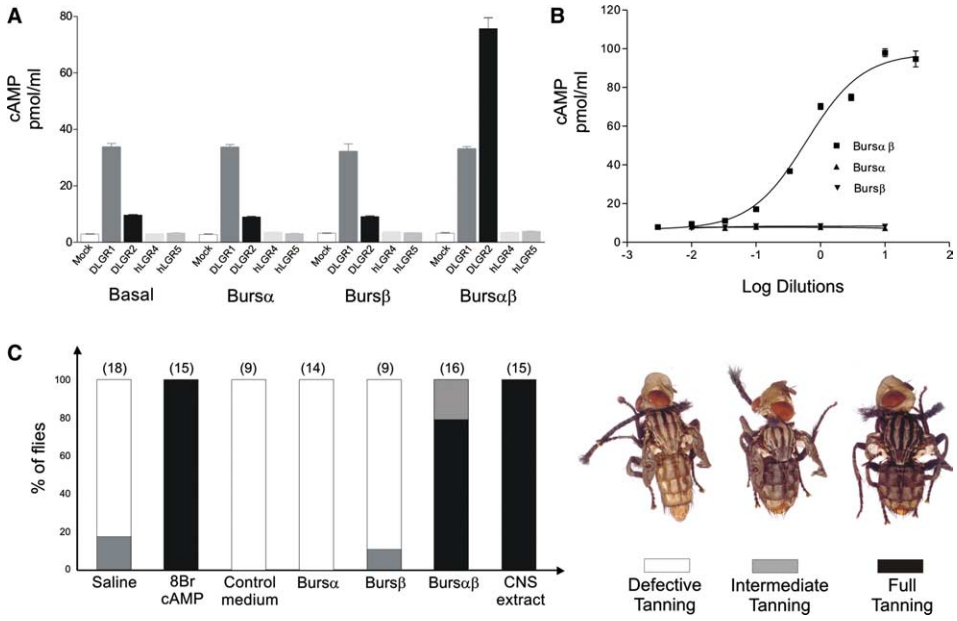

Fig. 2. Biological activities of *Drosophila* bursicon proteins. (A) Agonist activity on DLGR2. Conditioned media from control COS-7 cells (Basal) or cells expressing Bursα, Bursβ, or both proteins together (Bursαβ), were evaluated for their ability to induce cAMP accumulation in COS-7 cells transfected with *Drosophila* (DLGR1 and DLGR2) and human (hLGR4 and hLGR5) receptors (mock: cells transfected with empty plasmid). (B) Concentration-effect curve. Conditioned media were either diluted or concentrated (from 1/300 dilution to 30× concentration) to obtain serial concentrations of DLGR2 agonist activity. Error bars represent the range of duplicate determinations. (C) Bursicon bioassay. Saline, 8Br-cAMP, CNS extract or conditioned media from COS-7 cells transfected with empty plasmid (control medium) or from cells expressing Bursα, Bursβ, or both (Bursαβ) were injected into decapitated or neck-ligated, freshly eclosed flesh flies (*Neobellieria bullata*) to analyse their capacity to stimulate cuticular tanning. The number of flies tested per condition is given in parentheses. White, grey and black bars represent the percentage of flies showing defective, intermediate (abdomen) or full (thorax + abdomen) tanning, respectively. At the right side, photographs of flies representative of each tanning pattern.

(CG15284, hereafter called Bursβ). We identified the corresponding orthologous sequences in mosquito (*A. gambiae*), silkworm (*Bombyx mori*) and honey bee (*A. mellifera*) genomes, and manually predicted the corresponding ORFs (Fig. 1A). The encoded proteins are 72%, 63% and 65% identical to *Drosophila* Bursβ, respectively.

3.3. In the honey bee Bursα and Bursβ are encoded in a single ORF

Whereas the fly, mosquito and silkworm CG15284-like (Bursβ) sequences are predicted to be of similar length (141, 153 and 137 residues, respectively), the bee sequence constitutes the aminoterminal portion of a larger conceptual protein (269 aminoacids, Fig. 1A). It harbours a second cystine knot domain at its C-terminus, suggesting that it originated from the intragenic duplication of an ancient motif. In Fig. 1B, we show both cystine knot domains of the bee protein and the two Drosophila Bursα and Bursβ proteins, aligned with the cystine knot consensus sequence (CTCK: C-terminal cystine knot). Unexpectedly, detailed inspection of this second cystine knot domain, revealed that it corresponds to the bee ortholog of Bursα (CG13419) (Fig. 1A). In the other available insect species, Bursα and Bursβ are clearly encoded in distinct loci. Considering that a multidomain protein, encoded in a single gene in one species, may correspond to a multimeric protein, with each subunit encoded by separate genes, in another species [24–26], we hypothesized that *Drosophila* bioactive bursicon would be a heterodimer made of CG13419 and CG15284 subunits.

3.4. Co-expression of recombinant Drosophila Bursα and Bursβ generates bursicon bioactivity and DLGR2 stimulating activity

To test this hypothesis, Bursβ cDNA was cloned in the expression vector pcDNA3.1 and transfected in COS-7 cells, either alone or together with Bursα plasmid. Conditioned media were prepared from cultures of transfected cells and tested for their ability to stimulate cAMP accumulation in cells expressing DLGR2. Cells transfected with the cDNA of vertebrate or invertebrate orphan receptors belonging to the same subfamily of GPCRs (hLGR4, hLGR5 and DLGR1) [27,28] were used as controls. As can be seen in Fig. 2A, an 8-fold increase in cAMP production was induced in DLGR2-expressing cells, only when stimulated with conditioned medium from cells co-transfected with Bursα and Bursβ cDNAs. Stimulation with serial dilutions of the medium generated the expected sigmoid concentration–action curve (Fig. 2B). The conditioned media from cells transfected with Bursα or Bursβ plasmids alone had no effect (Fig. 2A). No stimulation was observed neither with a mixture made of equal amounts of media from cells transfected separately with Bursα or Bursβ (not shown). This indicates that, in order to achieve DLGR2 agonistic activity, Bursα and Bursβ need to be co-expressed in the same cell. DLGR1, a *Drosophila* receptor for a still unknown ligand [28], displayed strong constitutive activity [20] but was not stimulated. Neither were the human hLGR4 and hLGR5 receptors (Fig. 2A).

The identity between bursicon and the material present in the medium of cells co-transfected with *Drosophila* Bursα

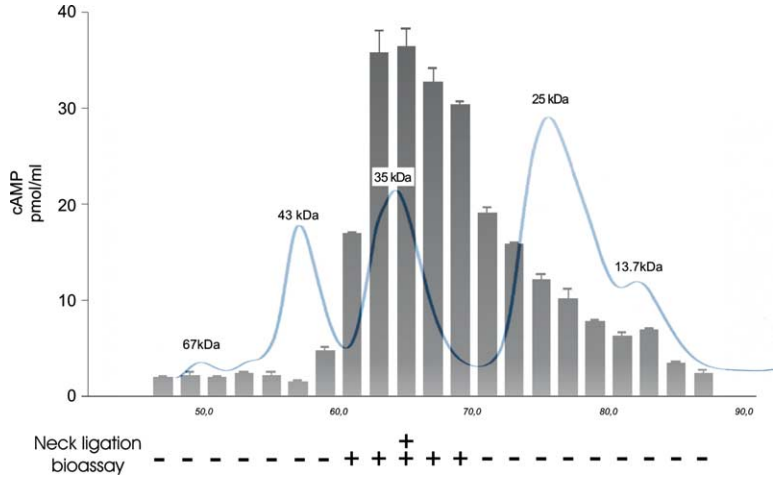

Fig. 3. Molecular weight estimation. Twenty times concentrated conditioned medium, produced in COS-7 cells co-transfected with Bursα and Bursβ cDNAs, was size fractionated by gel filtration chromatography. Eluted fractions between 70 and 10 kDa were analysed for their ability to stimulate cAMP accumulation in COS-7 cells expressing DLGR2. Grey bars represent the cAMP value after subtraction of the cAMP level of non-stimulated DLGR2 cells. The chromatogram (blue line) corresponds to 280 nm absorbance curve of the proteins used as molecular weight markers (67 kDa: Albumin; 43 kDa: Ovalbumin; 35 kDa: β-Lactoglobulin; 25 kDa: Chymotrypsinogen; 13.7 kDa: Ribonuclease A). Numbers in abscissa indicate the volume eluted from the column (ml). Error bars represent the standard error of four independent cAMP values obtained from each fraction. The lower panel (neck-ligation bioassays) shows the effects of injecting the fractions in neck-ligated flies (−, no tanning response; +, intermediate tanning; ++, full tanning; $n = 3$ per fraction).

and Bursβ cDNAs was established by microinjection in freshly eclosed, decapitated or neck-ligated *Neobellieria* (*Sarcophaga*) *bullata* flesh flies (Fig. 2C). Strong tanning of the cuticle was observed 3 h post-injection in flies injected with medium from COS-7 cells co-expressing Bursα and Bursβ. This response was also observed in flies injected with CNS extract derived from freshly eclosed flesh flies or with 8Br-cAMP, a membrane permeable cyclic AMP analog previously shown to mimic bursicon activity [13,14]. However, no bursicon activity was observed in flies injected with saline, control medium or media derived from cells transfected with Bursα or Bursβ cDNA alone.

3.5. *Drosophila* Bursicon is a heterodimer made of Bursα and Bursβ cystine knot subunits

The molecular weight of the bioactive molecule present in the conditioned medium was estimated by size-exclusion chromatography (Fig. 3). Eluted fractions were evaluated for bursicon bioactivity and their capacity to stimulate cAMP accumulation in DLGR2 expressing cells. Both activities co-eluted in the 30–35 kDa region of the chromatogram (Fig. 3). These results are in agreement with the expected molecular weights of Bursα and Bursβ protomers (15.5 and 13.8 kDa, after signal peptide removal, respectively), with the size reported for bioactive hormone [15], and demonstrate the heterodimeric nature of bursicon.

3.6. Bursα and Bursβ mRNAs are expressed at the same developmental stages

The expression of Bursα and Bursβ transcripts in *Drosophila melanogaster* was explored by RT-PCR at various stages of development. Both transcripts were found in embryonic, larval and pupal stages, but not in the adult of the fruit fly (Fig. 4). This pattern of expression of Bursα and Bursβ transcripts during development correlates with the one previously reported for DLGR2 [19].

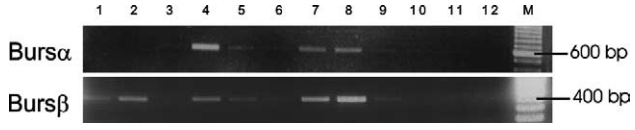

Fig. 4. Expression of Bursα and Bursβ transcripts in different developmental stages of the fruit fly, *Drosophila melanogaster*, as shown by PCR on normalized cDNA samples (see Section 2). Lanes 1–4: embryonic stages (0–4/4–8/8–12/12–24 h), lanes 5–7: larval stages 1–3, lane 8: pupal stage, lanes 9–10: adult head (males and females), lanes 11–12: adult body (males and females), M: marker (100 bp ladder).

4. Conclusion

We have demonstrated that the molting neurohormone bursicon is a heterodimeric protein made of two cystine knot subunits, and the natural agonist of the previous orphan G protein-coupled receptor DLGR2. A similar situation exists in vertebrates, where the pituitary hormones FSH, LH and TSH are also heterodimeric cystine knot proteins acting on their respective leucine-rich repeats containing receptors. The orphan GPCRs LGR4, LGR5 and LGR6 are putative orthologs of DLGR2 in vertebrates [29]. The knowledge of bursicon structure might help in the identification of candidate agonists for these orphan receptors, amongst the large family of cystine knot proteins. The two bursicon protomers, Bursα and Bursβ, are encoded by separate genes in *Drosophila*, *Anopheles* and *Bombyx*, but they are fused in a single gene in *Apis*. The sequence similarity between Bursα and Bursβ and their presence as a predicted single-chain protein in *A. mellifera* is a strong indication that they originated by duplication of (or in) an ancestral gene encoding a cystine knot protein.

From a practical point of view, our study makes it possible to develop screening assays for low molecular weight leucine-rich repeats containing G protein-coupled receptor 2 (LGR2) antagonists. These might qualify as effective novel agents for insect pest management.

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